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In vitro evaluation of the decontamination effect of cold atmospheric argon plasma on selected bacteria frequently encountered in small animal bite injuries

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Cold atmospheric argon plasma on selected bacteria

Abstract

Beneficial effects of cold atmospheric argon plasma (CAAP) on wound healing and its capacity for bacterial decontamination has recently been documented. First, *in vivo* studies in small animals did not prove any decontamination effect in canine bite wounds. The present study evaluated the overall decontamination effect of CAAP for different bacteria frequently encountered in canine bite wounds with respect to growth phase, initial bacteria concentration and treatment duration.

Standard strains of *Escherichia (E.) coli*, *Staphylococcus (S.) pseudintermedius*, *S. aureus*, *Streptococcus (S.) canis*, *Pseudomonas (P.) aeruginosa* and *Pasteurella multocida* were investigated. To evaluate the influence of the bacterial growth phase, each bacterium was incubated for three and eight hours, before CAAP treatment. Three different bacterial concentrations were created per bacterium and growth phase, and were exposed to CAAP for 30 seconds, 1 minute and 2 minutes.

CAAP treatment resulted in acceptable decontamination rates (range 98.9-99.9%) in all bacteria species *in vitro*; however, differences in susceptibility were detected. Decontamination rate was mainly influenced by initial bacterial concentration and treatment time. Growth phase only influenced decontamination in *S. pseudintermedius*. Treatment time significantly ($P<0.05$) correlated with the decontamination rate in *E. coli*, *S. canis* and *S. aureus*, with an exposure time of 2 minutes being most effective. Initial bacterial concentration significantly ($P<0.05$) influenced decontamination in *Pasteurella multocida* and *P. aeruginosa,* in which treatment time was not as important. CAAP exerts effective antibacterial activity against the tested bacteria strains in vitro, with species specific effects of treatment time, growth phase and concentration.

Keywords: cold atmospheric argon plasma, bacterial decontamination, time-dependent effects, concentration-dependent effects, bacterial growth

Introduction

Microbial multidrug-resistance (MDR) is one of the main issues that must be solved by modern medicine (Ogeer-Gyles et al. 2006, Weese 2008). Among surgical patients, surgical site infections, and especially open wounds, represent risk groups, and antibiotic treatment frequently results in a shift to more resistant bacteria rather than in wound decontamination (Harbarth 2007, Weese 2008, Nolff et al. 2016). Recognition of this problem has increased research on alternative strategies for wound decontamination, including antiseptic substances and physical treatment options in human medicine (Kramer et al. 2004, Assadian 2007). However, despite the fact that the same problem exists in small animal surgical patients (Weese 2008, Nolff et al. 2016), there is a paucity of studies focusing on antiseptic wound treatment in veterinary medicine (Sanchez et al. 1988, Lozier et al. 1992).

Plasma is a physical state of matter, which is highly energetic. A multimodal effect has been postulated, with the microbiocidal effect mainly attributed to reactive oxygen radicals (ROS), reactive nitrogen radicals (RNS), energetically enriched ions and charged particles, ultraviolet (UV) radiation and a mix of other atoms (Montie et al. 2000, Laroussi 2002, Laroussi and Leipold 2004, Matthes et al. 2014, Von Woedtke et al. 2013, Mai-Prochnow et al. 2014).

Various effects of cold atmospheric plasma on bacteria have been documented. Among these, three major impacts lead to cell destruction (Vatansever et al. 2013, Mai-Prochnow et al. 2014):

1. Destruction of the bacterial cell membrane
2. Intracellular protein damage
3. Direct DNA damage

Loaded particles, such as ions and electrons, create an electrostatic field that permeates the bacterial cell wall (Mendis et al. 2000, Laroussi et al. 2002, Von Woedtke et al. 2013). The electrostatic force accumulates on the cell surface until it causes the outward directed cell wall force to subside, leading to destruction (Mendis et al. 2000, Laroussi et al. 2002, Mai-Prochnow et al. 2014). This effect is more profound in Gram-negative bacteria, due to their more irregular rough outer membrane. While morphologic cell wall destruction has been documented in this Gram-negative bacteria species, no morphological changes occur in Gram-positive bacteria (Mendis et al 2000, Laroussi et al. 2002). Thus, less electrostatic force is needed to destroy their outer cell wall compared to Gram-positive bacteria (Mendis et al. 2000, Laroussi et al. 2002, Mai-Prochnow et al. 2014).

In addition, oxidative stress exerts direct effects on proteins and bacterial DNA (Laroussi et al. 2009, Von Woedtke et al. 2013). Massive oxidative stress through reactive oxygen radicals results in lipid-oxygenation, loss of cellular cytoplasm, proteins and oxidative damage to the DNA, leading to cell death once the reparation mechanism is overcome (Gadri et al. 2000, Imlay 2003, Joshi et al. 2011, Gaunt et al. 2006, Farr and Kogoma 1991). Prolonged exhibition to a plasma effluent results in complete cell fragmentation (Joshi et al. 2011). Finally, the cells undergo morphological and chemical changes before inactivation (Joaquin et al. 2009, Zhang et al. 2013). The described effects are dose-dependent and individually differ between target cells (Daeschlein et al. 2010, Fluhr et al. 2012, Matthes et al. 2013, Flynn et al. 2015).

Numerous *in vitro* and *in vivo* trials in small mammals and humans have documented an effective decontamination effect combined with a wide safety margin regarding a damage of eukaryotic cells (Laroussi et al. 2003, Daeschlein et al. 2010, Hammann et al. 2010, Ermolaeva et al. 2011, Koban et al. 2011, Fluhr et al. 2012, Lademann et al. 2012, Bundscherer et al. 2013, Matthes et al. 2013, Wende et al. 2014, Flynn et al. 2015, Kisch et al. 2016, Matthes et al. 2016).

Because the effect of plasma is related to numerous factors, a unique 'dosing' protocol is not available (Laroussi 2009, Von Woedtke et al. 2013). The type of effluent is closely related to the carrier gas, the room air and the device used for generation of the plasma state (Laroussi 2009, Von Woedtke et al., 2013). Furthermore, the distance between the plasma source and the wound surface influences the decontamination effect (Taghizadeh et al. 2015). Longer treatment times have been observedto result in greater 'bacterial death' however, this is influenced by bacteria species, growth form (biofilm) and substrate on which the bacteria grows (Laroussi et al. 2002, Cooper et al. 2010, Cotter et al. 2011, Brelles-Marino 2012, Taghizadeh et al. 2015).

Other factors, such as growth state (exponential or stationary phase), have been studied before by Yu et al. (2006) and initial bacterial concentration was evaluated in context of food microbiology. Fernandez et al. (2012) could show, that inactivation of *Salmonella Typhimurium* through CAP treatment is more efficient, the lower the initial bacterial concentration is. Despite these well-investigated facts, the first clinical trials evaluating the decontaminating effect of CAAP in canine bite wounds were not able to detect any decontamination effects in vivo (Winter et al. 2018). Therefore, it was our aim to investigate the effect of the used CAAP source in bacteria to be anticipated in canine bite wounds *in vitro* to document the in vitro efficacy and potential influencing factors with respect to bacteria species, bacteria growth phase and bacteria concentration. Based on a recent survey on the bacterial bio-burden of open treated wounds in small animal patients, the following bacteria were chosen for evaluation: *Escherichia (E.) coli*, *Staphylococcus (S.) aureus*, *Staphylococcus (S.) pseudintermedius*, *Streptococcus (S.) canis*, *Pasteurella multocida* and *Pseudomonas (P.) aeruginosa* (Nolff et al. 2016).

Our hypothesis was that CAAP would efficiently decontaminate all chosen isolates with an exposure time of 2 minutes being most effective. Furthermore, we postulated that this effect would be independent from the growth phase of the tested bacterium, and that higher bacterial

concentrations would need longer exposure times for decontamination. The aim was to investigate the efficiency of CAAP in vitro on selected bacteria before using it in a following *in vivo* study on canine bite wounds.

Materials and methods

Bacteria selection and culture

For this investigation, the following bacteria were included for further validation: *Escherichia coli* DSM 1103, *Staphylococcus aureus* DSM 1104, *Staphylococcus pseudintermedius* DSM 21284, *Streptococcus canis* DSM 20715, *Pasteurella multocida* DSM 5281 as well as *Pseudomonas aeruginosa* DSM 1117. All strains were obtained from the Leibniz Institute DSMZ German Collection of Microorganisms and Cell Cultures. To exclude the potential influence of different genetic alterations in wild-type bacteria, standard strains were chosen.

Bacterial suspensions were prepared using a standard-I-bouillon (No. 1.07882.0500, Merck) at a pH of 7.5 ± 0.2 at 37°C and plated on Mueller-Hinton Agar (No. 1.05435.0500, Merck). Because of growth requirements of the fastidious S. canis and *Pasteurella* multocida, Mueller-Hinton Agar was supplemented with 5% sheep blood. Sheep blood was drawn from in-house donors (animal house Schwere Reiter Straße 9, 80797 Munich, sheep blood donor registration nr. 55.2-1-54-2532.0-64-2016, government of upper Bavaria).

Determination of the bacterial growth phase

Bacterial proliferation in an closed system can be divided into four phases: A lag phase with no cell division, an exponential growth phase with high metabolic activity and cell division followed by a stationary phase, and the death phase. A wound is like an open system where all phases of bacterial proliferation can occur. For this study we only used the exponential growth and stationary phase for examination. In order to detect differences of the effect of CAAP with respect to the growth phase of the investigated bacteria, the transition from exponential

to stationary phase of each bacterium was determined by visual control using the McFarland standard. Each enrolled bacterium was incubated in standard I-bouillon at 37°C ± 1°C for 48 hours with a visual control every 2 hours to document the increase of turbidity until the stationary phase was reached (no more detectable increase). McFarland standard was used to simplify and keep scope of the trial down. Based on the results of the detected change in turbidity, an incubation of 3 hours was representative of exponential growth in all tested bacteria, while an incubation of 8 hours was representative of the stationary phase. Additionally, CFU´s were counted to confirm the exponential and stationary phase.

Determination of initial bacterial concentration

For determination of the colony-forming units per square centimetre (CFU/cm^2) a log10 serial dilution of the inoculum for plating was used. Colony counting was done after 48 hours of incubation at 37°C ± 1°C. For treatment with CAAP, three concentrations (10^{-1}, 10^{-2} and 10^{-3}) were plated per strain and per growth phase (6 plates per strain) on Mueller-Hinton Agar. After treatment with cold argon plasma, the plates were incubated at 37°C ± 1°C before counting colonies in the treated areas.

Cold Argon Plasma Treatment

The plasma device used in this study (KinPen Vet-NeoPlas) was an atmospheric plasma jet with a handheld unit, and consists of a 1 mm pin-tip electrode mounted in the centre of a quartz capillary (1.6 mm inner diameter, Figure 1 A). Argon was selected as a working gas at a flow of 5 standard litres per minute. The gas is ignited at the tip of the electrode and creates a jet-like effluent covering approximately 1 cm². The device consumes 50 W, operated at 230 V/50-60 Hz. The direct voltage within the plasma is 3 kV and has a dissipated electric capacity of 1.5-2 W. At these settings, the effluent has a visible length of 14 mm (Figure 1 B) and a constant temperature of 48°C at the tip.

To assure repeatability of treatment, a template was created and placed on the backside of each plate for plasma irradiation (Figure 2). Three areas of 1 cm² each were treated with the plasma device at an approximate distance of 1 cm above the surface for the duration of 30 seconds, 1 minute or 2 minutes. The KinPen Vet was therefore moved manually over each area in a meandering pattern with parallel lines and with different angles (top-down, from left to right, top right at lower left and top left at lower right). This pattern was repeated in concern for each given treatment duration.

After the plasma treatment, plates with isolates of the six bacterial strains were incubated for 14 to 20 hours at 37°C ± 1°C. *S. canis* was incubated for 44 to 46 hours due to the small size of colonies detected, in order to facilitate easier identification of CFUs. Experiments were repeated independently for each bacterial strain with all settings of the experiment. Three different log10 dilutions for each bacterial proliferation were plated respectively on agar plates. Each agar plate had three areas for CAAP treatment, achieving 18 areas for single examination of one bacterial strain. Altogether, 36 areas (n=36) for one and 216 areas (n=216) for all six different bacterial strains were examined. This resulted in different concentrations between the two tests of the bacterial strains and also for each growth phase.

Calculation of the decontamination effect

The CFU per plasma treatment zone of 1 cm^2 was divided by the CFU per 1 cm^2 without treatment, calculated from the log10 dilution of the inoculum. The values were given in percentage of growth reduction as well as in logarithmic growth reduction. In case CFU could not be counted after CAAP treatment, appraisal value was used referring to the untreated area of the agar plate. An 8 log reduction has been used for calculation in terms of a 100 % reduction. These results have been marked by * within the tables.

Statistics

Data were computed in Excel (Microsoft Excel 2010) and analysed using SPSS (IBM SPSS Statistics 23.0) software. Descriptive statistics were given as mean and standard deviation. Effect of treatment with respect to the investigated factors was determined using multivariate analysis of variance (ANOVA, SS Type III) and Fisher's f-test. Treatment time of CAAP, bacterial proliferation and initial bacterial concentration were tested regarding their effects on the decontamination. Significance was set at $P<0.05$.

Results

Decontamination effect with regard to treatment time

Treatment time represents one of the major aspects of CAAP efficiency. The effect of treatment time especially influenced the decontamination of *E. coli*, *S. aureus* and *S. canis*. In all three bacterial species, significant effects could be detected (Table 1). However, improved bacterial decontamination was observed in all investigated species at both growth phases. The most efficient treatment duration was 2 minutes per cm². Minor effects of treatment time were observed for *E. coli*, *P. aeruginosa* and *Pasteurella multocida* (Table 1). The influence of treatment time in *S. aureus* was superior to the effect of bacterial concentration.

In addition to total decontamination, another effect was observed: treatment duration correlated with the size of the inhibited area. The zone of inhibition exceeded the border of the treated area, despite the fact that all areas were treated based on the template. Extended treatment time, especially 2 minutes per cm², increased the zone of inhibition of all tested bacteria (Figure 3).

Decontamination effect with regard to bacterial growth phase

The decontamination rate per growth phase and bacterial strain is shown in Table 2. Higher decontamination rates were achieved in growing bacteria of *Pasteurella multocida*, *P. aeruginosa*, *S. aureus* and *S. pseudintermedius* as opposed to their stationary forms. However,

this difference was only significant for *S. pseudintermedius* (P= 0.001). Only *S. canis* showed a slightly higher decontaminating effect in the stationary form. In *E. coli,* no difference was detectable between the two bacterial proliferation phases.

Decontamination effect with regard to initial bacteria concentration

The decontamination rate per initial concentration and bacteria species is given in Table 3. With the exception of *E. coli*, bacteria concentration significantly influenced decontamination in all tested bacteria. This implies that with higher dilution, more effective decontamination is achieved through lower bacteria concentration.

Furthermore the effect of initial concentration (P= 0.000) seems to be higher compared with the growth phase (P= 0.001) of *S. pseudintermedius.* In *S. canis*, initial concentration (P= 0.001) has a greater effect compared to treatment time (P= 0.009).

Decontamination effect with regard to bacteria species

The overall logarithmic growth reduction of bacteria in all treated areas (n=216) ranged between log 2.56 and 5.39, in percentage terms between 71.4 and 99.9%. Treatment of Gram-negative bacteria (*E. coli* 99.99% = 5.39 log (n=36), *Pasteurella multocida* 96.65% = 4.76 log (n=36), *P. aeruginosa* 99.6% = 4.54 log (n=36)) resulted in an improved decontamination compared to Gram-positive species (*S. canis* 93.4% = 3.37 log (n=36), *S. aureus* 82.1% = 2.86 log (n=36) and *S. pseudintermedius* 71.4% = 2.56 log (n=36)).

An overview of initial bacterial concentration of each bouillon, CFUs before and after treatment for each serial dilution, treatment time and proliferation phase of respective bacteria species is given in Table 4.

Discussion

The aim of this study was to investigate the *in vitro* efficiency of CAAP on selected bacteria frequently encountered in canine bite wounds. Based on the overall effect, CAAP treatment seems to represent an interesting alternative to antiseptic wound treatment, especially since no known mechanisms for bacterial resistance have been documented in the literature (Heinlin et al. 2011, Daeschlein et al. 2012a, Daeschlein et al. 2015). Flynn et al. (2015) even documented a high efficiency against so-called ESKAPE bacteria (***E****nterococcus faecium,* ***S****taphylocoocus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa,* ***E****nterobacter spp.*), a group of pathogens frequently encountered in hospital-acquired infections in humans and animals (Weese 2008, Flynn et al. 2015, Nolff et al. 2016). However, the first study evaluating its effect in bite wounds in dogs did not find any decontaminating effect *in vivo* at all (Winter et al. 2018). Based on the beneficial results in experimental and human applications, this finding seems peculiar. We therefore wanted to evaluate the effect of the plasma source used *in vivo* in dog bite wounds in an *in vitro* setting to evaluate the general efficacy. We were able to prove that several factors impact CAAP performance, including initial bacterial contamination, treatment duration, bacterial growth phase and bacterial species.

The bacterial contamination of wounds is something that cannot be influenced through the clinician as it occurs before the patient is presented for medical treatment. Nevertheless, this study underlines the importance to reduce the contamination rate of wounds as far as possible before application of CAAP in order to achieve the maximum effect. In a clinical scenario, this can be achieved by debridement and pre lavage, which should always be performed based on our *in vitro* results.

As previously reported, we could detect a superior decontamination effect on Gram-negative bacteria compared with Gram-positive isolates. Laroussi et al. (2003) had previously detected superior effects in Gram-negative bacteria. They found that CAP treatment induced cell wall destruction, leading to loss of integrity and cytoplasm was distributed in clumps in Gram-

negative bacteria compared to untreated controls. In contrast, no differences between treated and untreated Gram-positive bacteria were detectable (Laroussi et al. 2003). These findings are in concordance with findings documented by Matthes et al. (2013), who reported lower decontamination rates for *S. epidermidis* compared to *P. aeruginosa*. The accumulation of the electrostatic force created by the CAP treatment is more crucial in Gram-negative species. This effect has been linked to their irregular rough outer membrane (Mendis et al. 2000, Montie et al. 2000, Laroussi et al. 2002). In contrast Lu et al. (2014) described the loss of cell integrity and additional DNA damage through time dependent CAP treatment and indicated that gram positive bacteria are more sensitive for inactivation induced by CAP than Gram-negative bacteria. The attending cannot influence the contamination under clinical conditions, and frequently there is a mixed flora of Gram-negative and Gram-positive bacteria present (Nolff et al. 2016). Despite the influence of bacteria species, the overall effect is still profound. Thus, clinical usage without knowledge of the wound status might still be helpful. However, if the contamination is known, CAAP is especially useful in treating patients affected by Gram-negative bacteria. Gram-positive bacteria should not be disregarded for CAAP treatment because of relatively good decontaminating results.

Yu et al. (2006) investigated the influence of CAP on the mid and late exponential phase as well as the stationary phase of treated bacteria. The study showed up, that exponential phases of bacteria are generally not more susceptible than their stationary phases, even cells in the mid exponential phase showed up a higher resistance than the other ones to plasma treatment. Bacteria in the stationary phase are more resistant to inimical processes than bacteria in the exponential growth phase (Dodd et al. 1997). It seems logical, that improved metabolism, as can be encountered in growing germs, might be linked with increased susceptibility to CAP compared to a more resting, stationary phase. We found that this aspect is negligible for most Gram-positive and Gram-negative tested bacteria species, with the exception of *S. pseudintermedius,* in which growth significantly improved the CAAP effect. This finding is

interesting, especially as no such difference was detected in *S. aureus*. However, individual differences, even between strains of methicillin-resistant *S. aureus* (MRSA) have been recently described (Matthes et al. 2016).

We also detected an impact of bacteria concentration on decontamination in all tested bacteria despite *E. coli*, with higher concentrations being less susceptible than lower concentrations. This effect was especially profound in *S. pseudintermedius* and *S. canis*. Our results agree to those of Fernandez et al. (2012), who could equally confirm a higher efficiency of CAP at lower bacteria concentration. In contrast to bacteria species and growth phase, bacteria concentration can actually be influenced in clinical cases by debridement and intense lavage, which should be included to reduce the concentration and treatment time and increase the efficiency of CAAP in clinical situations.

Finally, it was observed that increased treatment time results in increased decontamination, with the first decontamination effects visible after 30 seconds and a treatment time of 2 minutes being most effective. This is in concordance with the results of Matthes et al. (2013), who described a time-dependent linear efficiency of decontamination between 30 seconds and 5 minutes (300 seconds). Dose represents a term that remains under debate for plasma applications (Von Woedtke et al. 2013). Several studies have defined treatment time as dose-dependent; however, the energy level and amount of energy transferred should ideally also be included in determining the effective dose (Von Woedtke et al. 2013). Furthermore, other adjunctive treatments to CAP can increase the effective dose. It has been proven that irradiation of saline solution with CAP induces ionisation in the liquid, lowers the pH and alters reactive species within the fluid, making it bactericidal (Von Woedtke et al. 2013). Thus, the addition of saline lavage before treatment might exert an even bigger effect than pure CAP treatment.

It is important to consider that CAP dose exerts an impact on bacteria as well as on host cells. Therefore, the maximum dose has to be limited below the threshold that becomes toxic for the

host (Von Woedtke et al 2013). It has been proven that lower doses of plasma increase angiogenesis in host tissue, while higher doses induce cell death (Laroussi 2009, Von Woedtke et al. 2013). Fortunately, the lethal dose for bacteria is much smaller than the lethal dose for host tissue cells, leaving a wide safety margin for application (Hammann et al 2010, Ermolaeva et al. 2011, Koban et al. 2011, Daeschlein et al. 2012b, Lademann et al. 2012, Bundscherer et al. 2013, Matthes et al. 2013, Wende et al. 2014, Kisch et al. 2016).

Based on the significant results achieved in this *in vitro* trial, we are not able to explain the poor performance of CAAP in the clinical situation. We were able to prove, that the used device in the used setting is capable of effectively killing the most frequently encountered bacteria types. However, the data of the current trial underline the importance of appropriate exposure time. The design of the KinPen Vet is adapted to clinical use, facilitating easy application and transportability. Unfortunately, the design allows only treatment of a relatively small area about 1 cm². The duration of treatment not only influences the rate of decontamination but also increases the decontaminated area. This effect has been previously described in CAP-treated culture wells and is hypothesized to be a reflection of the plasma jet at the surface of the well. Another potential explanation for this inhibitory effect around the actual treated area might include the diffusion and activation of oxygen radicals within the perimeter of the treated area (Joshi et al. 2010).

Therefore, a needed dose of 2 minutes per cm² might result in a long treatment time in clinical patients, prolonging anaesthesia time and thus increasing the risk for surgical site infections. We think that this effect represents the most important clinical limitation. If the results of the study by Winter et al. (2018) are interpreted under the light of our current findings, we think that the usage of bigger devices, which allow appropriate treatment time under clinical conditions, would most likely improve performance *in vivo*.

Conclusion

CAAP exerts a substantial decontamination effect on bacteria frequently found in animal bite injuries *in vitro*. Even if Gram-negative and positive bacteria are affected differently by CAP treatment, there is no formation of complete bacteria resistance (without any effect on bacteria), which is why this treatment might present an interesting alternative for local wound decontamination *in vivo*. However, bacteria concentration should initially be reduced as far as possible before initiation of CAAP treatment in clinical cases. Furthermore, adequate treatment time must be ensured.

Conflict of interest statement

NeoPlas Greifswald supplied the KinPen Vet used in this study. NeoPlas played no role in the study design, data collection, analysis and interpretation of data, or the decision to submit the manuscript for publication. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Figure legends.

Figure 1. Schematic (A, figure courtesy of INP Greifswald) and life (B) view of the KinPen Vet handheld device.

Figure 2. Template with three areas of 1 cm^2 each for CAP treatment for 30 seconds, 1 minute or 2 minutes.

Figure 3. Effect of treatment time (arrowhead, 30 seconds, black arrow, 1 minute, *, 2 minutes) on different concentrations (left row 10^-1, middle row 10^-2 and right row 10^-3 bacteria) of *Escherichia coli* (A), *Staphylococcus aureus* (B), *Staphylococcus pseudintermedius* (C), *Streptococcus canis* (D), *Pasteurella multocida* (E) as well as *Pseudomonas aeruginosa* (F) during the growth phase.

Conflict of interest statement

NeoPlas Greifswald supplied the KinPen Vet used in this study. NeoPlas played no role in the study design, data collection, analysis and interpretation of data, or the decision to submit the manuscript for publication. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Table 1. Decontamination rate with respect to treatment time

Bacteria species	Mean decontamination rate							
	% decontamination (each n=12)			Log reduction (each n=12)				
	0.5 min	1 min	2 min	0.5 min	1 min	2 min	P-value	η^2
E. coli	99.98	99.99	100	4.52	5.74*	5.92*	0.001	0.286
P. multocida	89.97	99.98	99.99	3.97	5.15	5.16	0.096	0.084
P. aeruginosa	98.71	99.99	100	3.64	4.37	5.59*	0.227	0.045
S. canis	83.11	97.09	99.96	2.49	3.66	3.95	0.009	0.195
S. aureus	72.25	79.98	97.90	1.85	2.54	4.18	0.001	0.291
S. pseudintermedius	67.85	68.19	78.31	2.29	2.40	2.99	0.413	0.021

*8 log reduction is used for calculation

Table 2: Decontamination rate with respect to bacterial growth phase

Bacteria species	Mean decontamination rate					
	% decontamination (each n=18)		Log reduction (each n=18)			
	exponential	stationary	exponential	stationary	P-value	η^2
E. coli	99.99	99.99	5.31*	5.48*	0.944	0.000
P. multocida	97.20	96.10	4.68	4.84	0.879	0.001
P. aeruginosa	99.98	99.14	4.53*	4.54*	0.718	0.004
S. canis	92.49	94.28	3.09	3.64	0.373	0.025
S. aureus	87.66	79.09	3.18	2.54	0.428	0.020
S. pseudintermedius	84.15	58.75	3.31	1.81	0.001	0.295

*8 log reduction is used for calculation

Table 3: Decontamination rate with respect to bacterial concentration

Bacteria species	Mean decontamination rate							
	% decontamination (each n=12)			Log reduction (each n=12)				
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	P-value	η^2
E. coli	99.99	99.99	99.99	6.75*	4.88	4.55	0.127	0.071
P. multocida	89.98	99.98	99.98	4.43	5.09	4.51	0.004	0.234
P. aeruginosa	98.74	99.98	99.97	5.15*	4.40	4.06	0.004	0.228
S. canis	80.80	99.55	99.81	2.27	4.08	3.74	0.001	0.282
S. aureus	72.08	78.30	99.76	1.53	2.57	4.47	0.021	0.157
S. pseudintermedius	30.98	84.56	98.81	0.92	3.13	3.63	0.000	0.352

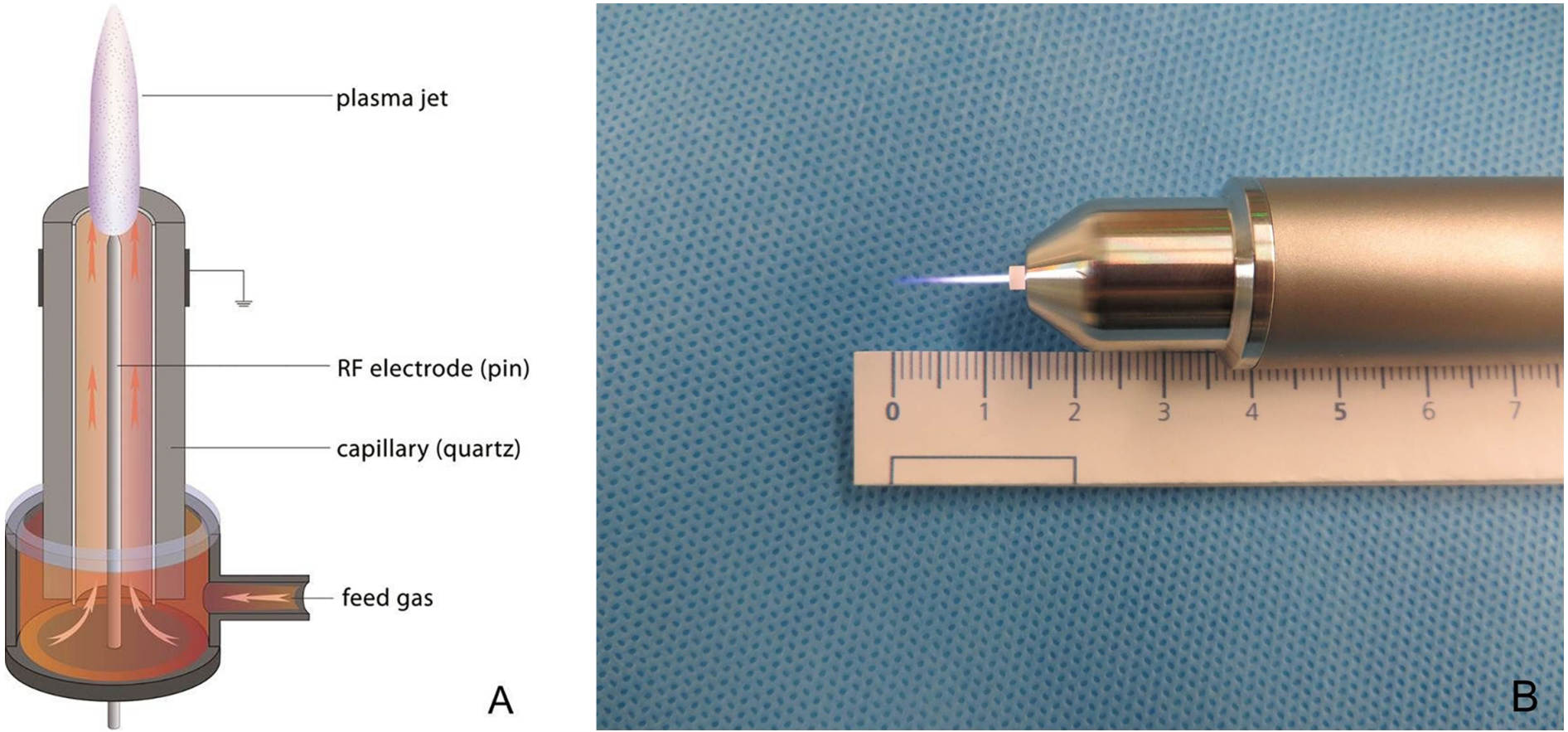
*8 log reduction is used for calculation

Table 4. Overview of countable CFU's before and after cold argon plasma treatment with initial CFU per ml and CFU per cm^2

Bacteria species	Trial	Incubation time of bouillon (hours)	CFU/ml inoculum bouillon (0-value)	CFU/ml for agar plate (after 3 h / 8 h of incubation)	CFU/cm^2 for cold argon plasma treatment (per area)	Countable CFU's in area after treatment (for serial dilution and treatment time)								
						10^{-1} and 30 sec	10^{-1} and 1 min	10^{-1} and 2 min	10^{-2} and 30 sec	10^{-2} and 1 min	10^{-2} and 2 min	10^{-3} and 30 sec	10^{-3} and 1 min	10^{-3} and 2 min
E. coli	1	3	12 x 10^{-6}	31 x 10^{-7}	53 x 10^{-4}	2	0	0	2	0	0	1	0	0
		8	73 x 10^{-5}	64 x 10^{-7}	11 x 10^{-5}	24	0	0	6	0	0	2	0	0
	2	3	29 x 10^{-6}	25 x 10^{-7}	43 x 10^{-4}	37	0	0	3	0	0	1	0	0
		8	32 x 10^{-6}	83 x 10^{-7}	14 x 10^{-5}	14	2	0	3	0	0	2	0	0
P. multocida	1	3	31 x 10^{-6}	33 x 10^{-7}	57 x 10^{-4}	4	4	5	8	6	2	5	1	0
		8	26 x 10^{-6}	67 x 10^{-7}	12 x 10^{-5}	5	2	3	4	8	3	0	0	6
	2	3	69 x 10^{-6}	12 x 10^{-8}	21 x 10^{-5}	*	19	12	15	3	1	12	3	0
		8	4 x 10^{-6}	1 x 10^{-8}	18 x 10^{-5}	*	14	9	6	0	5	4	2	0
P. aeruginosa	1	3	69 x 10^{-6}	11 x 10^{-7}	18 x 10^{-4}	14	5	0	5	2	0	0	0	0
		8	19 x 10^{-6}	12 x 10^{-7}	21 x 10^{-4}	41	8	0	1	5	0	1	0	0
	2	3	79 x 10^{-6}	11 x 10^{-7}	18 x 10^{-4}	24	19	0	8	0	0	0	1	0
		8	42 x 10^{-6}	33 x 10^{-7}	57 x 10^{-4}	*	25	0	27	0	0	7	1	0
S. canis	1	3	16 x 10^{-6}	14 x 10^{-7}	24 x 10^{-4}	*		41	18	11	8	10	2	0
		8	13 x 10^{-6}	95 x 10^{-6}	16 x 10^{-4}	*	50	17	20	6	2	2	0	6
	2	3	12 x 10^{-5}	14 x 10^{-6}	24 x 10^{-3}	*	20	18	3	83	2	1	3	0
		8	36 x 10^{-4}	14 x 10^{-7}	24 x 10^{-4}	*	16	33	93	211	4	21	14	5
S. aureus	1	3	21 x 10^{-7}	26 x 10^{-7}	45 x 10^{-4}	*	*	*	*	*	20	60	26	2
		8	13 x 10^{-7}	37 x 10^{-7}	64 x 10^{-4}	*	*	*	*	*	30	50	50	4
	2	3	16 x 10^{-7}	27 x 10^{-7}	47 x 10^{-4}	*	30	1	100	1	0	13	0	0
		8	77 x 10^{-6}	72 x 10^{-7}	12 x 10^{-5}	*	*	100	*	*	3	30	12	0

S. pseudintermedius	1	3	77×10^{-6}	11×10^{-7}	18×10^{-4}	*	*	*	65	33	24	16	6	0
		8	11×10^{-7}	27×10^{-6}	47×10^{-3}	*	*	*	*	*	20	24	24	4
	2	3	80×10^{-6}	94×10^{-6}	16×10^{-4}	83	60	14	15	36	2	1	0	2
		8	47×10^{-6}	3×10^{-6}	52×10^{-3}	*	*	*	120	*	72	35	30	2

* CFU's not countable under visual control


plasma jet
RF electrode (pin)
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A
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Figure 1

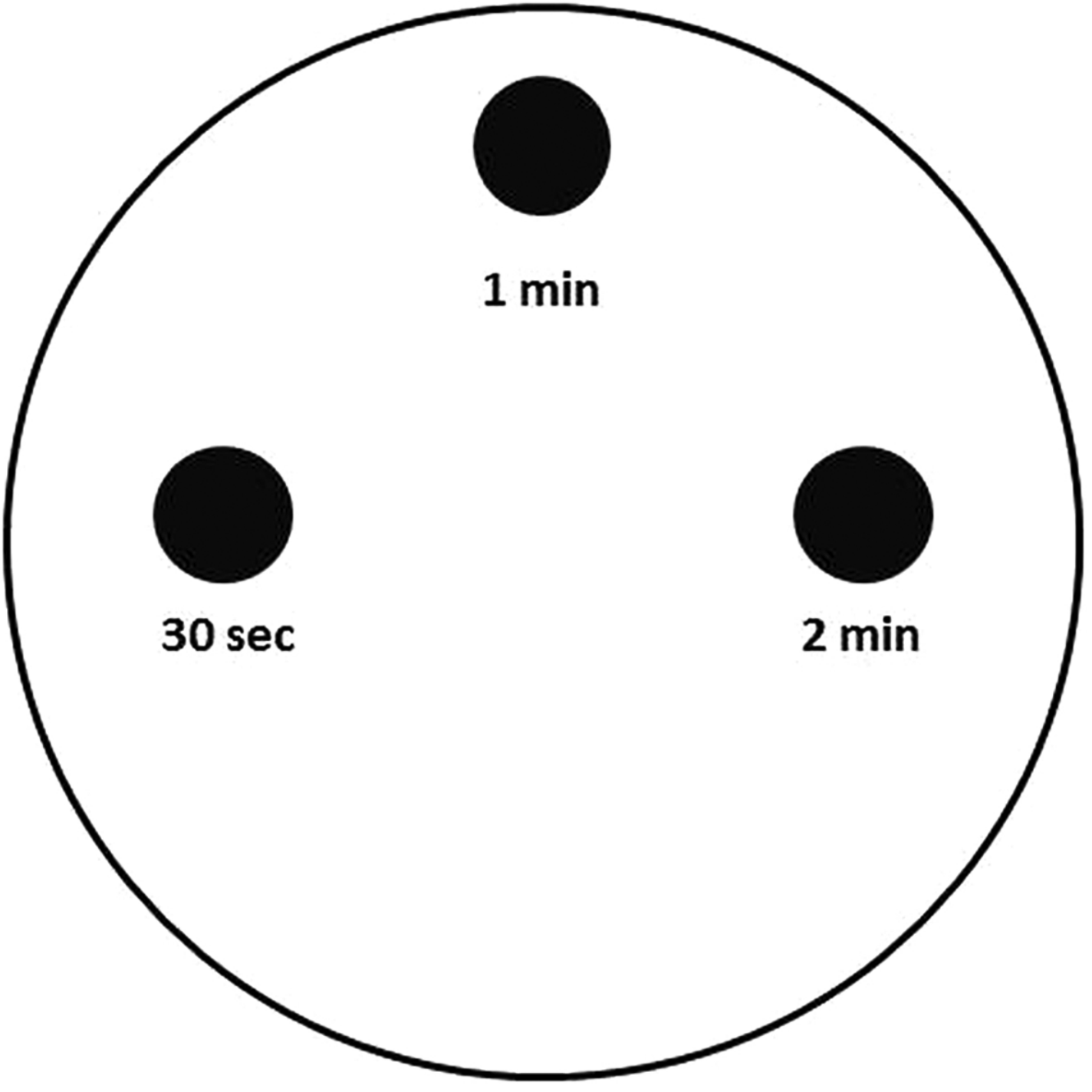

Figure 2

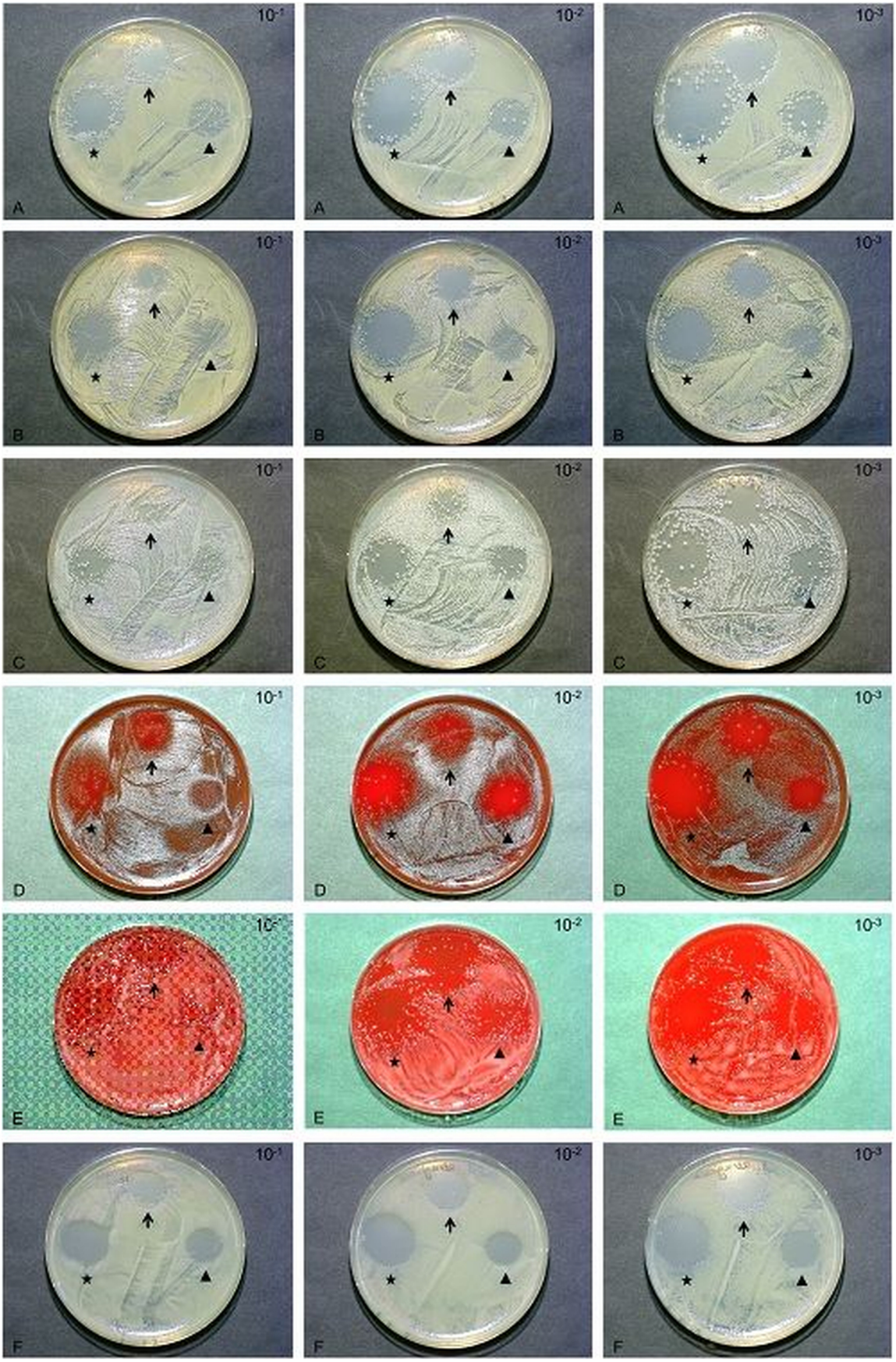

Figure 3